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Short communication

Neospora caninum detected in feral rodents

M.C. Jenkins [a,*], C. Parker [a], D. Hill [a], R.D. Pinckney [b], R. Dyer [c], J.P. Dubey [a]

[a] *United States Department of Agriculture, Agricultural Research Service, Animal Parasitic Diseases Laboratory, Building 1040, BARC-EAST, Beltsville, MD 20705, USA*
[b] *Department of Paraclinical Studies, School of Veterinary Medicine, St. George's University, Grenada, West Indies*
[c] *Department of Animal and Food Sciences, 531 South College Avenue, 050 Townsend Hall, University of Delaware, Newark, DE 19717, USA*

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Abstract

The role of rodents in the epidemiology of neosporosis was investigated by assaying brain tissue of feral mice (*Mus musculus*) and rats (*Rattus norvegicus*) for *Neospora caninum*. Both mouse and rat brain tissue were extracted for total DNA, and subjected to two different *N. caninum*-specific nested polymerase chain reaction (PCR) assays. A portion of brain tissue from the mice and rats were also assayed for *N. caninum* in gerbils or gamma-interferon gene knockout (KO) mice. Of the 105 feral mice tested, 10% were positive in the *N. caninum*-specific PCR assays. Of the 242 rats tested, 30% were positive in both assays. Although mice and rats had *N. caninum* by PCR testing, clinical signs of *N. caninum* infection were not observed nor were *N. caninum* parasites observed in gerbils or KO mice inoculated with the rodent brain tissue.

Keywords: *Neospora caninum*; Rats (*Rattus norvegicus*); Mice (*Mus musculus*); PCR; Bioassay

1. Introduction

Neosporosis is a protozoan parasitic disease that appears to be a major cause of abortion in dairy cattle worldwide (Dubey, 2005). The causative agent, *Neospora caninum*, is passed efficiently from dam to fetus, such that the majority of calves born from seropositive dams are themselves infected with the parasite (Dubey, 2003; Innes et al., 2000). *N. caninum* is transmitted either in utero (endogenous transmission) or by ingestion of oocysts that have been shed by canids (exogenous transmission), the definitive host of the parasite (McAllister et al., 1998). The role of dogs in the epidemiology of neosporosis is supported by the finding of an association between the presence of dogs and the incidence of neosporosis on dairy farms (Dijkstra et al., 2002; Wouda et al., 1999). The discovery of *N. caninum* in a variety of wild animals, including deer and coyotes, suggests that a sylvatic cycle may play a role in the epidemiology of this disease (Dubey and Thulliez, 2005; Gondim et al., 2004a; Rosypal and Lindsay, 2005). At present, there have been only two reports of *N. caninum* in rodents (Huang et al., 2004; Hughes et al., 2006). The purpose of the present study was to test whether feral mice and rats harbor *N. caninum*, and thus may serve as a potential source of the parasite in the environment.

2. Materials and methods

2.1. Feral mice (*Mus musculus*)

From June to October 1999, a total of 105 mice were live caught using aluminum folding live capture traps

* Corresponding author. Tel.: +1 301 504 8300; fax: +1 301 504 5306.
E-mail address: mjenkins@anri.barc.usda.gov (M.C. Jenkins).

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(Forestry Supplies Inc., Jackson, MS, USA) set around feed bunks, loafing sheds, dairy cow barns, feed storage areas, hay lofts and milking parlors on 2 Delaware dairy farms that experienced chronic abortions due to neosporosis. The mice were transported in the traps to the laboratory in Beltsville, MD, USA, and killed by exposure to CO_2 gas. The mice were necropsied by first removing blood from the thoracic vena cava for analysis of anti-*Neospora* antibodies using the *N. caninum* agglutination test (NAT, Romand et al., 1998), and then removing the brain for PCR analysis and bioassay. One portion of the brains was placed in DNA extraction buffer, while another portion was placed in a sterile test tube and incubated on ice for inoculation into gerbils using standard procedures (Dubey et al., 2004).

2.2. Feral rats (Rattus norvegicus)

From April to December 2005 a total of 242 rats were caught using wire mesh traps set in different locations in the 6 Parishes of Grenada, West Indies (Dubey et al., in press). The rats were then transported to the Pathology Laboratory at the School of Veterinary Medicine, St. George's University, then anesthetized and killed with an over-dose of ether in a closed chamber. The thoracic cavity was opened and blood was collected from the heart for NAT assay. The rats were necropsied, and fresh samples of brain were taken for *N. caninum* isolation. All samples were immediately shipped on ice to the Animal Parasitic Diseases Laboratory, United States Department of Agriculture, Beltsville, MD, USA, for analysis.

2.3. Extraction of DNA and analysis by polymerase chain reaction

Mouse and rat brain tissue were extracted in DNA extraction buffer (Liddell et al., 1999) containing 0.1 mg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO, USA) and 0.1% sodium dodecylsulfate (SDS, Sigma Chemical Co.) by incubation overnight at 50 °C in a shaking water bath. Cross-contamination of DNA between individual mouse or rat brain tissues was eliminated by using autoclaved instruments that were thoroughly rinsed between samples by immersion in sterile water, 100% ethanol, 2 M NaOH, followed by a final water rinse (Liddell et al., 1999). The tissues were extracted with successive phenol, phenol–chloroform and chloroform treatment, and the DNA concentrated by ethanol precipitation (Sambrook et al., 1989). The DNA was washed once with 70% ethanol, allowed to air dry, and then suspended in 500 μl 1 mM Tris, pH 8.3, 0.1 mM EDTA. The DNA concentration was estimated by measuring absorbance at OD_{260}. Mouse and rat brain DNA were then assayed for the presence of *N. caninum* by nested PCR directed at two different targets—ITS1 (Buxton et al., 1998) and Nc5 (Kaufmann et al., 1996; Liddell et al., 1999; Muller et al., 1996; Yamage et al., 1996). The primers (see Table 1) used for primary and nested PCR were identical to those described (Buxton et al., 1998; Yamage et al., 1996), except the nested Np10 primer, which was designed in-house. All primary and nested PCR amplifications were performed a minimum of two times for each mouse or rat brain DNA sample. Positive control samples consisted of brain tissue DNA from inbred mice infected with *N. caninum*; the negative control consisted of H_2O diluent. The amplification products were analyzed on 7.5% polyacrylamide mini-gels (BioRad Laboratories Inc., Hercules, CA, USA) electrophoresed at 100 V in 40 mM Tris, 30 mM $NaPO_4$, 1 mM EDTA, pH 7.9 buffer, using 100 bp DNA markers (BioRad) as size standards. The gels were stained with ethidium bromide and photographed on a 254 nm UV transilluminator using a CCD camera (Biophotonics Inc., Ann Arbor, MI, USA).

Table 1
DNA sequence of primary and nested ITS1 and Nc5 primers used for detection of *Neospora caninum* DNA in brain tissue of feral mice and rats

Primer name	Primary/ nested	DNA sequence (5′–3′)
ITS1 rDNA		
NN1[a]	Primary	TCAACCTTTGAATCCCAA
NN2[a]	Primary	CGAGCCAAGACATCCATT
NP1[a]	Nested	TACTACTCCCTGTGAGTTG
NP2[a]	Nested	TCTCTTCCCTCAACGCT
Nc5		
Np6+[b]	Primary	CTCGCCAGTCAACCTACGTCTTCT
Np21+[b]	Primary	GGGTGTGCGTCCAATCCTGTAAC
Np7[c]	Nested	GGGTGAACCGAGGGAGTTG
Np10	Nested	TCGTCCGCTTGCTCCCTATGAAT

[a] Primers identical to those described in Buxton et al. (1998).
[b] Primers identical to those described in Muller et al. (1996).
[c] Primers identical to those described in Yamage et al. (1996).

2.4. Bioassay of mouse and rat tissue for N. caninum

A portion of brain tissue from 80 of 105 wild mice was inoculated into gerbils using a standard procedure (Dubey et al., 2004). Brain tissue either from individual rats (*N. caninum* seropositive) or pooled from multiple rats (seronegative) were bioassayed in gamma-interferon gene knockout (KO) mice using standard

Table 2
Assay for *Neospora caninum* in feral mice and rats by detection of *N. caninum* in brain tissue using nested polymerase chain reaction (PCR) assays directed at ITS1 rDNA or Nc5 DNA sequences, serological testing of sera by *Neospora* agglutination test (NAT) and bioassay for *N. caninum* in gamma-interferon knockout (KO) mice or gerbils

Source of brain tissue	Percentage PCR positive for *N. caninum* (PCR positive/total)[a]			Seropositivity[a]	Bioassay[a]
	ITS1	Nc5	ITS1 + Nc5		
Mouse	21.9 (23/105)	9.5 (10/105)	8.6 (9/105)	5.1 (4/79)	0.0 (0/105)
Rat	43.8 (106/242)	68.6 (166/242)	39.7 (96/242)	4.6 (11/242)	0.0 (0/242)

[a] Values in each column represent the percentage positive by ITS1 nested PCR, Nc5 nested PCR, or positive in both ITS1 and Nc5 assays, or by NAT testing, or by bioassay in KO mice (for feral mouse brain tissue) or gerbils (for rat brain tissue).

procedures (Dubey et al., 2004). Preliminary evidence for *N. caninum* exposure was determined by NAT using a 1:20 dilution of sera from each rat. Brain tissue from all rats was bioassayed for *N. caninum* by homogenizing one-half brain tissue from each of the seropositive rats using a mortar and pestle in antibiotic saline, and inoculated into one to five KO mice. Brains of the remaining seronegative rats in each batch were pooled, incubated in 0.5% trypsin at 37 °C for 30 min, washed three times by centrifugation and the homogenates inoculated subcutaneously into two to five KO mice. Both the gerbils and KO mice were observed for 2 months, then bled and their sera were tested for *N. caninum* antibodies at a serum dilution of 1:25 in the NAT. Gerbils and KO mice were killed 2 months p.i. and their brain smears were examined for the presence of *N. caninum* tissue cysts.

3. Results

At least 10% of feral mice were found to contain *N. caninum* DNA in brain tissue as revealed by nested PCR (Table 2). There was incomplete agreement between the two assays with nested ITS1 PCR detecting *N. caninum* DNA in 21.9% (23/105) and Nc5 detecting 9.5% (10/105) wild mouse brain tissues. Mouse brain tissue that was positive in both assays was 8.6% (9/105). A much higher percentage of rat brains were *N. caninum* positive in both nested PCR assays; nearly 40% (96/242) were positive in both the ITS1 and Nc5 assays (Table 2). In contrast to the results obtained with feral mouse samples, the percentage positive rat brain samples were appreciably higher using the nested Nc5 assay (68.6%) compared to the nested ITS1 assay (43.8%).

Although nested PCR indicated the presence of *N. caninum* in 10% of wild mice and 40% of wild rats, no clinical signs of neosporosis appeared in nor were *N. caninum* tissue cysts or a serological titer to *N. caninum* observed in gerbils or KO mice that had been inoculated with feral mice or rat brain tissue. *N. caninum* antibodies were found in sera of eleven feral rats (in titers of 1:20 in four rats, 1:40 in four rats, 1:160 or higher in three rats) and in sera of four feral mice (in titers of 1:25 in one mouse, and 1:50 in three mice).

4. Discussion

This study provides preliminary evidence for *N. caninum* in the brains of feral mice and rats. Brain tissue from nearly 10% of mice and 40% of rats were shown to contain detectable levels of *N. caninum* based on the criterion of giving a positive amplification signal in both the ITS1 and Nc5 assays. If the criterion for positivity is the appearance of an amplification product in at least one assay, then the percentage of mice possibly harboring *N. caninum* in brain tissue is nearly 23%. Using this same criterion, over 70% (176/242) of rats would contain *N. caninum* in brain tissue.

It is unclear at present why there is not complete agreement between the two assays. It is possible that there is genotypic variability in ITS1 rDNA and Nc5 DNA sequences, such that either the primary or nested PCR primers do not anneal to the respective sequences. However, the primary and nested primer pairs for both sequences were chosen because of their conservation in the *N. caninum* genome, and the observed variability is outside of these primer sites (Gondim et al., 2004b; Muller et al., 2001). While there may be slight differences in sensitivities of the assays because both the ITS1 rDNA and Nc5 sequences exists in multiple copies (Gondim et al., 2004b; Slapeta et al., 2002), there was no consistent higher percentage posivitity with either assay.

Although PCR provided evidence for *N. caninum* in nearly 10% of feral mice and 40% of feral rats, antibodies to *N. caninum* were detected in only 4 feral mice (5.1%) and 11 feral rats (4.6%). The higher percentage of positive feral mice and rats as indicated by PCR compared to serology has precedent in a related cyst-forming protozoan, *Toxoplasma gondii*. One research group found that a proportion of rats which acquired *T. gondii* by

congenital infection as revealed by bioassay remained seronegative to *T. gondii* (Dubey et al., 1997). These authors speculated that congenital infection may lead to tolerance to the *T. gondii*. It is also unclear why no viable *N. caninum* tissue cysts or antibodies to *N. caninum* were detected in gerbils or KO mice inoculated with feral mice or rat brain tissue. One explanation for the absence of clinical signs, serological titers to *N. caninum*, or brain cysts may be that the parasites in brain tissue were not viable, but had intact DNA present. The time between trapping of feral mice and rats, and assay of brain tissue may have been too long to sustain the viability of tissue cysts. A number of other authors have identified *N. caninum* and *T. gondii* in brain tissue using PCR, but have been unable to culture viable parasites (Ferre et al., 2005). Whether *N. caninum* cysts in brains of wild mice and rats die more rapidly than in neural tissue of other animals is unknown.

Little is known concerning the sources of infection for the definitive (dog, coyote) hosts for *N. caninum* in the wild. Small mammalian species are the logical reservoir host but there is only scant information on this topic. For instance, there is a report of *N. caninum*, as revealed by serology (indirect fluorescent antibody test), in 9 of 55 *Rattus norvegicus* caught on 6 dairy farms in Taiwan (Huang et al., 2004). In this study, *N. caninum* DNA was detected in two of the seropositive rats by Nc5 PCR, and *N. caninum* was isolated from the brain of one of the two DNA positive rat brain by bioassay in nude mice (Huang et al., 2004). A single *N. caninum* tissue cyst was found in a nude mouse that had been inoculated 6 months earlier with the *N. caninum* positive rat brain. In another study, *N. caninum* DNA was detected in 3 of 100 mice and 2 of 45 rats from sheep farms in England (Hughes et al., 2006). Our results also provide further evidence that both feral mice and rats harbor *N. caninum* in brain tissue, and thus may serve as a source of the parasite to domestic and wild canids in the life cycle of *N. caninum*.

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